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GENETICS AND MOLECULAR BIOLOGY

Burkholderia thailandensis Methylated Hydroxyalkylquinolines: Biosynthesis and Antimicrobial Activity in Cocultures

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ABSTRACT The bacterium *Burkholderia thailandensis* produces an arsenal of secondary metabolites that have diverse structures and roles in the ecology of this soil-dwelling bacterium. In coculture experiments, *B. thailandensis* strain E264 secretes an antimicrobial that nearly eliminates another soil bacterium, *Bacillus subtilis* strain 168. To identify the antimicrobial, we used a transposon mutagenesis approach. This screen identified antimicrobial-defective mutants with insertions in the *hmqA*, *hmqC*, and *hmqF* genes involved in biosynthesis of a family of 2-alkyl-4(1*H*)-quinolones called 4-hydroxy-3-methyl-2-alkenylquinolines (HMAQs), which are closely related to the *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs). Insertions also occurred in the previously uncharacterized gene BTH_II1576 ("*hmqL*"). The results confirm that BTH_II1576 is involved in generating *N*-oxide derivatives of HMAQs (HMAQ-NOs). Synthetic HMAQ-NO is active against *B. subtilis* 168, showing ~50-fold more activity than HMAQ. Both the methyl group and the length of the carbon side chain account for the high activity of HMAQ-NO. The results provide new information on the biosynthesis and activities of HMAQs and reveal new insight into how these molecules might be important for the ecology of *B. thailandensis*.

IMPORTANCE The soil bacterium *Burkholderia thailandensis* produces 2-alkyl-4(1*H*)-quinolones that are mostly methylated 4-hydroxyalkenylquinolines, a family of relatively unstudied metabolites similar to molecules also synthesized by *Pseudomonas aeruginosa*. Several of the methylated 4-hydroxyalkenylquinolines have antimicrobial activity against other species. We show that *Bacillus subtilis* strain 168 is particularly susceptible to *N*-oxidated methylalkenylquinolines (HMAQ-NOs). We confirmed that HMAQ-NO biosynthesis requires the previously unstudied protein HmqL. These results provide new information about the biology of 2-alkyl-4(1*H*)-quinolones, particularly the methylated 4-hydroxyalkenylquinolines, which are unique to *B. thailandensis*. This study also has importance for understanding *B. thailandensis* secondary metabolites and has implications for potential therapeutic development.

KEYWORDS *Burkholderia*, cell-cell interaction, natural antimicrobial products, quinolones

The saprophytic betaproteobacterium *Burkholderia thailandensis* is closely related to two pathogens, *Burkholderia pseudomallei* and *Burkholderia mallei*, which are the causative agents of melioidosis and glanders, respectively (1, 2). *B. pseudomallei* is also a saprophyte and causes respiratory or skin infections in humans following exposure to organisms in the environment, such as through skin contact with soil (3). *B. mallei* is a

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host-adapted pathogen and is spread to humans from horses and other ungulates, in which it is endemic in some regions (4). Because *B. pseudomallei* and *B. mallei* are tier 1 select agents and require handling under biosafety level 3 (BSL-3) laboratory conditions, *B. thailandensis* is often used as a surrogate to study the biology and virulence mechanisms of these pathogens (5). The development of versatile genetic techniques (6–9) and improvements in mouse models of melioidosis (10) have greatly improved the ability to study the biology of this relatively understudied group.

There has been much interest in elucidating the arsenal of small molecules produced by *B. thailandensis*, where there are at least 13 polyketide synthesis (PKS) gene clusters, many of which are conserved in *B. mallei* and/or *B. pseudomallei*. Although many of these metabolites have now been identified, only a few have been studied in much detail. One of the best studied is bactobolin (11, 12), which blocks translation by binding to a unique site in the 50S ribosomal subunit (13). Other PKS antibiotics are malleilactone (14, 15) and malleicyprol, a more toxic product of the malleilactone biosynthetic gene cluster (16), which contribute to virulence of *B. pseudomallei* (17). *B. thailandensis* also produces thailandenes, a group of polyenes with activity against Gram-positive bacteria (18). As with many bacterial natural products, malleilactone and thailandenes are not produced under standard laboratory conditions (14, 15, 18). Studies of these molecules were possible through genetic (14, 15) or chemical (19) elicitation of the gene clusters or through phenotype-based screening approaches (18).

Most of the PKS gene clusters are unique to this group of *Burkholderia* species. A few of them have analogous biosynthesis pathways in other *Burkholderia* species or even beyond the *Burkholderia* genus. For example, the *hmqABCDEFG* operon coding for enzymes responsible for the biosynthesis of a family of 4-hydroxy-2-alkylquinolines (HAQs) named 4-hydroxy-3-methyl-2-alkenylquinolines (HMAQs) are found in *B. thailandensis*, *B. pseudomallei*, and other members of the *Burkholderia* genus, such as *Burkholderia ambifaria* (20). The products made by the HmqABCDEFG enzymes have differing carbon chain lengths and saturation and presence of substitutions on the quinolone ring, such as methylation and oxidation. The relative abundances of these various congeners differ between species (21). The *hmq* operon is homologous to the *pqs* operon found in *P. aeruginosa* (Fig. 1) (21, 22). The molecules produced by *Burkholderia* also differ from those of *P. aeruginosa* in that most bear a methyl group at the 3′ position and possess an unsaturated aliphatic side chain, which are linked to the presence of the additional *hmqG* and *hmqF* genes, respectively (21). The main product of the *P. aeruginosa pqs* operon, 4-hydroxy-2-heptylquinoline (HHQ), is converted to 3,4-dihydroxy-2-heptylquinoline (Pseudomonas quinolone signal [PQS]) by the enzyme PqsH (23, 24). Both HAQs are involved in quorum sensing in *P. aeruginosa* and are detected by the MvfR (PqsR) regulator (25–27). No homologs of the *pqsH* and *mvfR* genes have been found in *Burkholderia* species (21).

We are interested in the small-molecule repertoire of *B. thailandensis* as an avenue to better understand the biology of this bacterium and make new discoveries in natural product biosynthesis. We observed that *B. thailandensis* culture fluid has significant antimicrobial activity that is not due to bactobolin, the only other known antimicrobial produced under these conditions. This bactobolin-independent activity was isolated to the *hmq* gene cluster using an approach involving transposon mutagenesis and screening for mutants exhibiting reduced antimicrobial activity. Purified and synthetic HAQ derivatives were assessed for the antimicrobial properties of several such biosynthetic products of the *hmq* genes, including HMAQ congeners and *N*-oxide derivatives (HMAQ-NOs) with various alkenyl side chain lengths. We also confirmed the involvement of *hmqL* in the biosynthesis of HMAQ-NO compounds. Our results provide new information on the biosynthesis and activities of the HMAQs produced by *Burkholderia*.

RESULTS

Antimicrobial activity of *B. thailandensis* bactobolin-null mutants. Initial liquid coculture experiments with *B. thailandensis* strain E264 and *B. subtilis* strain 168 (hereinafter referred to as *B. thailandensis* or *B. subtilis*, respectively) showed that *B.*

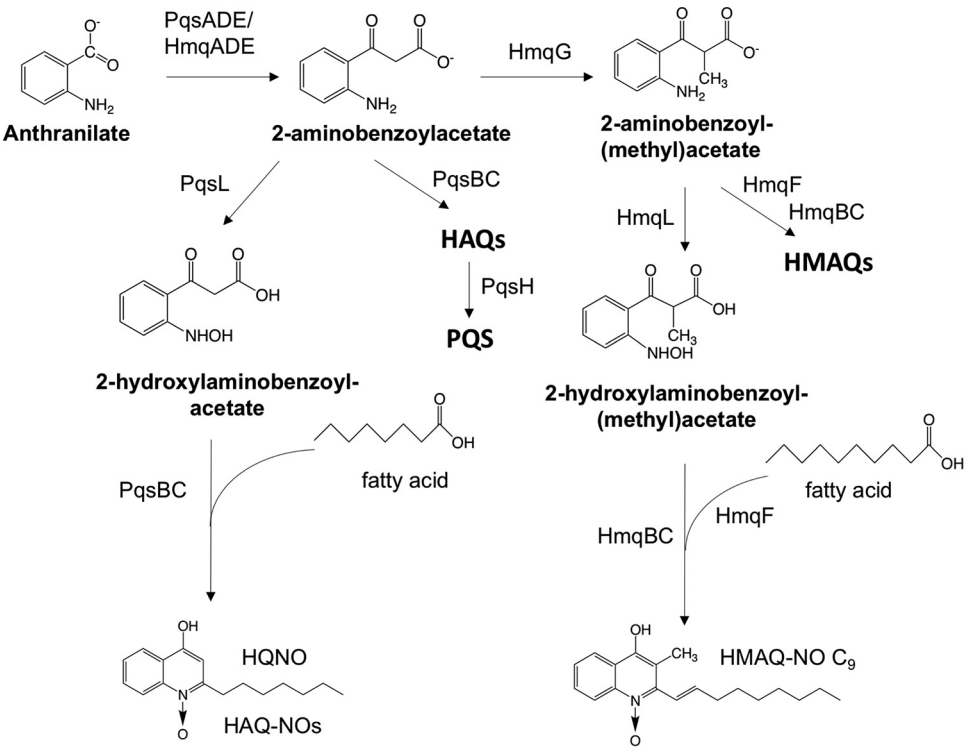

FIG 1 Biosynthesis of 4-hydroxy-2-alkylquinoline congeners. *Burkholderia thailandensis* uses the *hmq* gene products to synthesize HAQs including HMAQ and HMAQ-NO. In *Pseudomonas aeruginosa*, the analogous *pqs* gene products synthesize the related compounds HAQ, HAQ-NO, and PQS. Shown are the *N*-oxidated species referred to in the text, HQNO and HMAQ-NO-C_9 with a double bond at the 1′-2′ position added by HmqF. The *B. thailandensis* compounds are methylated by HmqG, which does not have a homolog in *P. aeruginosa*. PqsH is needed for production of PQS, which is specific to *P. aeruginosa*.

thailandensis has a strong growth advantage over *B. subtilis*. The growth advantage was so substantial that after overnight liquid coculture with *B. thailandensis*, *B. subtilis* decreased from a density of 10^6 cells per ml to below the limit of detection ($<10^2$ cells per ml). This result was not solely attributable to bactobolin, as a bactobolin-null mutant (BD20) also had the same growth advantage over *B. subtilis* (Fig. 2A). This observation led to the hypothesis that *B. thailandensis* has a previously uncharacterized antimicrobial activity against *B. subtilis* that is not mediated by bactobolin. To further explore this hypothesis, culture fluids of several *B. thailandensis* strains were harvested and tested for antimicrobial activity (Fig. 2B). As previously observed (11), filter-sterilized culture fluids of wild-type *B. thailandensis* saturating a paper filter disc placed on a lawn of *B. subtilis* caused a zone of growth inhibition around the filter disc, whereas there was no growth inhibition observed with the bactobolin-null BD20 strain (Fig. 2B, top panel). However, unprocessed culture fluid of both strains (wild type and BD20), which had not gone through the filter sterilization process, demonstrated antimicrobial activity (Fig. 2B, middle and bottom). This observation (i.e., that only unprocessed culture fluid had bactobolin-independent antimicrobial activity) could be explained by several possible hypotheses: first, that the filter sterilization process removes or inactivates antimicrobial activity, and second, that antimicrobial activity requires live cells. In support of the first hypothesis, the antimicrobial activity was observed in the absence of viable *B. thailandensis* bactobolin mutant cells; unprocessed *B. thailandensis* BD20 culture fluid had activity against *B. subtilis* when added directly to high-salt LB agar plates, which are conditions that do not allow *B. thailandensis* growth (Fig. 2B, bottom). Ethyl acetate extracts of *B. thailandensis* cultures also had activity against *B. subtilis* (Fig. S1 in the supplemental material). Together, these results suggest that *B. thailandensis* produces an antimicrobial other than bactobolin, which is eliminated by filter sterilization.

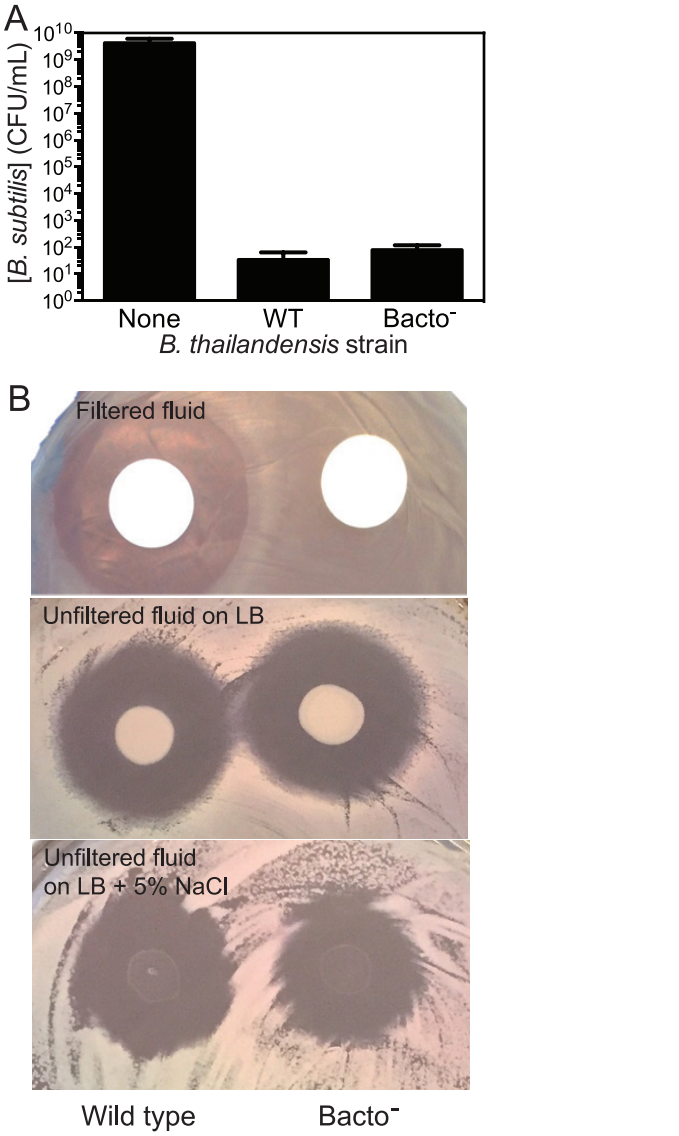

FIG 2 Sensitivity of *Bacillus subtilis* strain 168 to a substance produced by *Burkholderia thailandensis*. (A) For liquid coculture growth, *B. subtilis* strain 168 was combined in a 1:1 ratio with either *Burkholderia thailandensis* E264 (WT) or bactobolin-deficient *B. thailandensis* (Bacto$^-$, strain BD20) in LB broth and grown for 24 h at 37°C prior to plating to determine surviving CFU as described in Materials and Methods. Data are representative of three biological replicates. Error bars show standard deviations. (B) Growth inhibition of *B. subtilis* strain 168 following treatment with cultures or culture fluid from *B. thailandensis* after 18 h of growth on plates. *B. thailandensis* wild type (E264) or the bactobolin-defective mutant (Bacto$^-$, strain BD20) was applied to a lawn of freshly plated *B. subtilis* 168, and plates were incubated at 30°C prior to imaging. Top, *B. thailandensis* culture fluid was filtered and used to saturate paper diffusion discs applied to the lawn of *B. subtilis* 168. A zone of clearing around a diffusion disc indicates the region where *B. subtilis* growth was inhibited. Results are similar to those previously reported (11). Middle, unfiltered *B. thailandensis* fluid (10 μl) was spotted directly onto *B. subtilis* 168. Bottom, unfiltered *B. thailandensis* fluid was spotted onto a lawn of *B. subtilis* 168, as in the experiment whose results are shown in the middle panel, but on medium containing 5% NaCl, which inhibits *B. thailandensis* growth.

Isolation and identification of antimicrobial-deficient transposon mutants. To identify the genes required for the observed antimicrobial activity, we used a mutagenesis and screening approach. First, we randomly mutagenized the *B. thailandensis* bactobolin-null mutant BD20 with a transposon containing the trimethoprim resistance gene *dhfR* (Tn*5*::*dhfR*). Next, we screened the mutants (~10,000) using a high-throughput method to assess antimicrobial activity (for experiment overview, see Fig. S2). Briefly, we added *B. subtilis* cells to cooled molten agar and mixed gently before

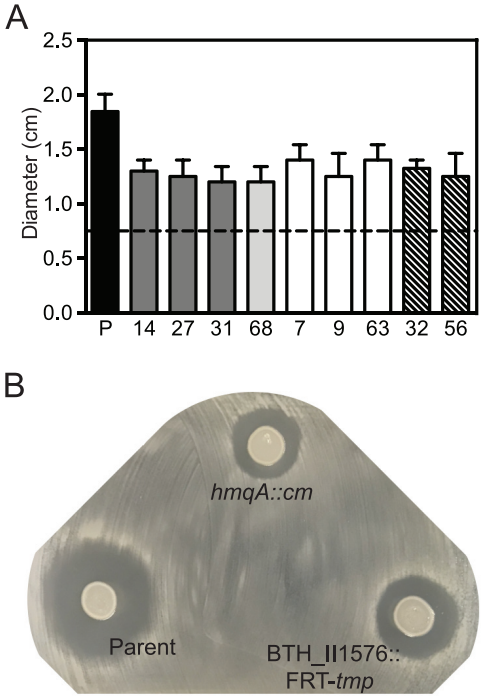

FIG 3 *B. thailandensis* transposon mutants with reduced effects on *Bacillus subtilis* strain 168. (A) Amounts of 5 μl of unfiltered fluid from *B. thailandensis* stationary-phase cultures were spotted onto lawns of freshly plated *B. subtilis* strain 168 and incubated overnight at 30°C. Results are shown as the diameters of the zones of inhibition. The black dashed line indicates the diameter of the spot of *B. thailandensis* culture. Transposon mutant numbers correspond with those in Table 1 and are shaded according to the gene mutation, as follows: dark gray, *hmqA* disruptions; light gray, *hmqC* disruption; white, *hmqF* disruptions; hatched, BTH_II1576 disruptions. P (parent), *B. thailandensis* bactobolin-deficient mutant BD20 used for transposon mutagenesis. Data are the averages of two biological replicates. Error bars show standard deviations. (B) Images of *B. subtilis* 168 lawns spotted with 5 μl unfiltered fluid from cultures of the *B. thailandensis* bactobolin-deficient strain BD20 (Parent) or BD20 with a disruption in *hmqA* (*hmqA*::*cm*) or BTH_II1576 (BTH_II1576::FRT-*tmp*) introduced by homologous recombination.

pouring into plates. After the medium solidified, single isolated colonies (i.e., transposon mutants) were patched onto the plates. The next day, plates were assessed for zones of inhibition. *B. thailandensis* patches demonstrating reduced zones of inhibition compared with the *B. thailandensis* bactobolin-defective parent were reisolated for further study. We initially identified 60 antimicrobial-defective candidates. Of those, 9 were confirmed to have reduced antimicrobial activity against *B. subtilis* (Fig. 3A) with no observable growth defects (Table S1). These were mutants 7, 9, 14, 27, 31, 32, 56, 63, and 68 (Table 1).

TABLE 1 Locations of transposon insertions

Mutant	Locus	Gene	Predicted gene function	Insertion location (gene length)[a]
14	BTH_II1935	*hmqA*	2-Aminobenzoate-CoA ligase	1231 (1,626)
27	BTH_II1935	*hmqA*	2-Aminobenzoate-CoA ligase	1513 (1,626)
31	BTH_II1935	*hmqA*	2-Aminobenzoate-CoA ligase	1478 (1,626)
68	BTH_II1933	*hmqC*	Unknown	783 (1,107)
7	BTH_II1930	*hmqF*	Polyketide synthase	163 (3,972)
9	BTH_II1930	*hmqF*	Polyketide synthase	2524 (3,972)
63	BTH_II1930	*hmqF*	Polyketide synthase	2872 (3,972)
32	BTH_II1576	"*hmqL*"[b]	Putative monooxygenase	226 (1,167)
56	BTH_II1576	"*hmqL*"	Putative monooxygenase	998 (1,167)

[a]The number of the nucleotide just prior to the transposon insertion relative to the predicted translation start site is given, followed by the total nucleotides in the gene in parentheses.
[b]*hmqL*, proposed name from reference 22.

TABLE 2 Antimicrobial activities of hydroxylalkylquinoline analogs

Quinolone family[a]	Carbon chain	$[M + H]^+$	MIC (μg/ml)[b]	
			B. subtilis 168	*S. aureus* Newman
HMAQ	$C_{9:2'}$	284	50	25
HMAQ-NO	$C_{9:2'}$	300	0.75	25
HAQ (HHQ)	C_7	242	>200	>200
HAQ-NO (HQNO)	C_7	259	25	25
HMAQ-NO	$C_{8:2'}$	286	1.5	6.25
HMAQ-NO	$C_{7:2'}$	272	6.25	12.5

[a]HMAQ with a C_9 carbon chain (HMAQ-$C_{9:2'}$) was purified as described in reference 21. HMAQ-NO congeners were synthesized as described in Materials and Methods and Piochon et al. (31). HAQ-NO with a C_7 carbon chain (HQNO) and HAQ with a C_7 carbon chain (HHQ) were purchased from commercial sources (Cayman Chemicals and Sigma Aldrich, respectively).

[b]No activity of any of the compounds against *Pseudomonas aeruginosa* strain PA14 or *Escherichia coli* strain JM109 was observed up to 200 μg/ml. Results are the averages of three independent experiments. In all cases, the range was <5%.

The locations of the transposon insertions were identified using whole-genome resequencing of five of the mutants and PCR amplification of suspected target gene loci of the remaining four (see Materials and Methods). Of the nine mutants isolated, seven had insertions in the *hmqABCDEFG* operon (BTH_II1929 to -1935) (Table 1). The other two mutants had disruptions in a previously unstudied gene, BTH_II1576, which is predicted to encode a monooxygenase. To verify that the *hmq* locus and BTH_II1576 contribute to the antimicrobial defects observed for the transposon mutants, we disrupted *hmqA* or BTH_II1576 in the bactobolin-defective BD20 strain using homologous recombination. Both gene disruptions caused defects in *B. subtilis* growth inhibition similar to those observed with the transposon mutants (Fig. 3B), supporting the idea that the *hmq* genes and BTH_II1576 are important for the bactobolin-independent antimicrobial activity of *B. thailandensis*.

Identification and activities of *hmq* gene products. Both the *pqs* and *hmq* gene products use anthranilic acid and fatty acid precursors to generate HMAQs and HAQs through the pathway illustrated in Fig. 1. The result of biosynthesis includes molecules with unsaturated or saturated side chains and *N*-oxide derivatives. The most abundant HMAQ in *B. thailandensis* E264 cultures is a congener with an unsaturated C_9 side chain, 4-hydroxy-3-methyl-2-nonenylquinoline, referred to as HMNQ or HMAQ-$C_{9:2'}$ (21). To test whether production of HMAQ-$C_{9:2'}$ is absent in our transposon mutants, we measured this HMAQ in culture fluid using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to find a product with the expected *m/z* of 284. Consistent with previous results (21, 28), transposon mutants with insertions in *hmqA* and *hmqF* had no HMAQ-$C_{9:2'}$ (<0.05 μg/ml, the limit of detection). We also detected no HMAQ-$C_{9:2'}$ in *hmqC* mutants, consistent with the proposed role of HmqC in HMAQ biosynthesis (Fig. 1). The BD20 parent strain and BTH_II1576 transposon mutants both readily produced this HMAQ congener (measured at 5 to 8 μg/ml).

We tested the activity of HMAQ-$C_{9:2'}$ directly against *B. subtilis* using a standard MIC assay. The MIC of purified HMAQ-$C_{9:2'}$ against *B. subtilis* was 50 μg/ml (Table 2). HMAQ-$C_{9:2'}$ also inhibited the growth of *Staphylococcus aureus* (MIC of 25 μg/ml). We did not detect any antimicrobial activity of HMAQ-$C_{9:2'}$ against *Escherichia coli* or *Pseudomonas aeruginosa* (MIC of >200 μg/ml). Of note, the concentration of HMAQ-$C_{9:2'}$ in *B. thailandensis* cultures (5 to 8 μg/ml) is ~5-fold lower than needed to inhibit *B. subtilis* growth (50 μg/ml), suggesting that HMAQ-$C_{9:2'}$ alone is not sufficient for the observed effects on *B. subtilis* in our coculture experiments. Instead, we hypothesized that the antimicrobial activity in cocultures involved another product of the *hmq* genes.

Biosynthesis and antimicrobial activity of HMAQ-NO. The protein product of BTH_II1576 shares 52% amino acid sequence identity to the *P. aeruginosa* PqsL protein involved in HAQ biosynthesis. PqsL synthesizes 2-hydroxylaminobenzoylacetate (2-HABA) from 2-aminobenzoylacetate (2-ABA) as a step in the pathway to make *N*-oxide derivatives (HAQ-NOs) (Fig. 1, left column) (29, 30). We hypothesized that BTH_II1576 is

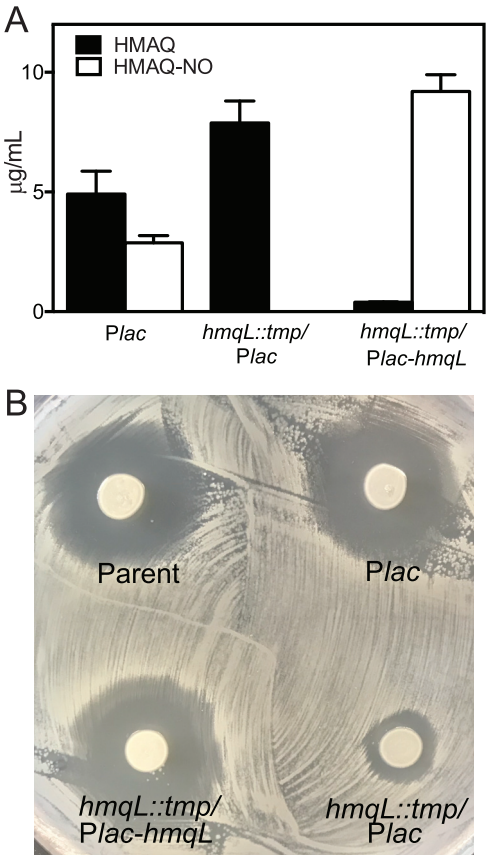

FIG 4 BTH_II1576 (*hmqL*) involvement in HMAQ-NO production and activity against *B. subtilis* strain 168. (A) HMAQ-NO (C_9) was quantified in stationary-phase *B. thailandensis* strains using LC-MS/MS and methods described previously (21). Error bars show standard deviations. (B) Antimicrobial activity of unfiltered *B. thailandensis* fluid (5 μl) on a lawn of freshly plated *B. subtilis* on plates containing 1 mM IPTG. Strains tested were the *B. thailandensis* bactobolin-deficient BD20 (Parent), BD20 with the IPTG-inducible P*lac* expression cassette inserted into the neutral *glmS1* site in the genome (BD20 P*lac*), the constructed BD20 BTH_II1576 (*hmqL*) mutant with the P*lac* cassette in *glmS1* (*hmqL*::*tmp*/P*lac*), or the BD20 *hmqL* mutant with P*lac-hmqL* in *glmS1* (*hmqL*::*tmp*/P*lac-hmqL*).

similarly involved in the biosynthesis of *N*-oxide HMAQ (HMAQ-NO) in *B. thailandensis* (Fig. 1, right column). To test this hypothesis, we used LC-MS/MS to measure HMAQ-NO in the BTH_II1576 transposon mutants. We measured HMAQ-NO with an unsaturated C_9 or C_7 side chain, which are two abundant HMAQ congeners in *B. thailandensis* E264 cultures. Both of the BTH_II1576 mutants and our constructed BD20 BTH_II1576 mutant had undetectable HMAQ-NO-C_9 ($<$0.05 μg/ml), whereas the BD20 parent had measurable levels (1.5 $\pm$ 0.5 μg/ml [mean $\pm$ standard deviation]). We also expressed BTH_II1576 ectopically from an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *lac* promoter in the neutral *glmS1* site in the engineered BTH_II1576 mutant genome, and we compared HMAQ-NO-C_9 and the antimicrobial activities in this strain with those of an empty *lac* promoter-containing mutant or BD20 parent (Fig. 4). IPTG induction of BTH_II1576 in the mutant restored the production of HMAQ-NO (Fig. 4A) and increased the zone of inhibition of *B. subtilis* in colony outgrowth experiments (Fig. 4B), supporting the idea that BTH_II1576 is important for each of these processes. Furthermore, BTH_II1576 induction significantly decreased HMAQs, supporting the idea that the product of BTH_II1576 uses HMAQ precursors as substrate to generate HMAQ-NO. Together, our results confirm that the BTH_II1576 product is analogous to PqsL in HAQ-NO biosynthesis and is appropriately named HmqL, as previously proposed (22).

Because HmqL generates HMAQ-NO and is important for the antimicrobial activity observed in *B. thailandensis* cultures, we tested the hypothesis that HMAQ-NO has antimicrobial activity against *B. subtilis*. We assessed the sensitivity

of *B. subtilis* to the most abundant HMAQ-NO produced by *B. thailandensis* (21), synthetic HMAQ-NO-$C_{9:2'}$ (31) (Table 2). The MIC of HMAQ-NO-$C_{9:2'}$ against *B. subtilis* was 0.75 μg/ml. This MIC is below the measured concentration of HMAQ-NO-$C_{9:2'}$ in *B. thailandensis* cell cultures (1.5 $\pm$ 0.5 μg/ml), supporting the idea that HMAQ-NO is primarily responsible for the observed antimicrobial activity against *B. subtilis* in cocultures with *B. thailandensis*. Interestingly, there was no difference in activity between the C_9 congeners of HMAQ-NO and HMAQ against *S. aureus* strain Newman (MIC 25 μg/ml). Differences in diffusion or target site availability could explain the differences in relative activities of these two molecules in each species.

Antimicrobial activities of structurally related 4-hydroxy-2-alkylquinolines. We found it intriguing that the C_7 HAQ (HHQ) and its *N*-oxide derivative HAQ-NO-C_7 (HQNO) were much less active against *B. subtilis* than the respective C_9 HMAQ and HMAQ-NO molecules (Table 2). The difference in activity could be due to the difference in alkyl chain lengths or saturation levels. Alternatively, the presence of the methyl group in HMAQs could also affect the activity. To address the first possibility, we tested synthetic HMAQ-NO congeners with a C_7 and a C_8 unsaturated alkyl side chain against *B. subtilis*. Our results showed that the C_8 and C_7 HMAQ-NO molecules were 2- and 8-fold less active, respectively, than the C_9 congener against *B. subtilis* (Table 2). These results suggest the molecule with the longer carbon chain length has higher activity of HMAQ-NO against *B. subtilis*. The C_7 HMAQ-NO was also more active than HQNO (HAQ-NO-C_7) by about 2-fold against the *S. aureus* Newman strain and 4-fold against *B. subtilis* (Table 2). HQNO differs from C_7 HMAQ-NO in that it is unmethylated and has a saturated side chain. Thus, either methylation or saturation of the side chain also plays a role in the activity.

HMAQ promotes competition in liquid cocultures. The results of our transposon mutant analysis suggest that HMAQs, and in particular HMAQ-NO-C_9, eliminate *B. subtilis* from liquid cocultures, providing an explanation for our initial observation. To test this hypothesis, we competed *B. subtilis* with a *B. thailandensis* bactobolin-deficient BD20 strain containing either a single *hmqA* or *hmqL* mutation or an *hmqA-hmqL* mutation in liquid coculture experiments. Singly disrupting *hmqA* nearly abolished *B. thailandensis* activity against *B. subtilis* (Fig. 5A). However, singly disrupting *hmqL* showed only a small and not statistically significant reduction of activity. A strain in which both *hmqL* and *hmqA* were disrupted showed defects in antimicrobial activity similar to that of the *hmqA* single mutant, supporting the idea that HmqA is upstream from HmqL in the biosynthetic pathway. These results show that the activity against *B. subtilis* in liquid cocultures is due to *hmq* biosynthetic products but not HMAQ-NO alone. The activity either requires HMAQ-NO together with other *hmq* biosynthetic products or is independent of HMAQ-NO.

Our initial observations suggested that the antimicrobial in *B. thailandensis* cultures was sensitive to filtration; thus, we also sought to test the sensitivities of HMAQ and HMAQ-NO to filtration. We measured the concentrations of each of these molecules in unfiltered and filtered fluid from cell-free *B. thailandensis* cultures. We also determined the concentrations of these molecules in pelleted cells to determine if they are primarily associated with the cell, similar to HAQs in *P. aeruginosa* (26, 32). We found that the percentages of HMAQs and HMAQ-NOs in the cell fraction, which included molecules associated with the cell membrane, periplasm, and cytoplasm, were 91% $\pm$ 2% and 71% $\pm$ 3%, respectively. Thus, these molecules are highly cell associated. Furthermore, filtration further depletes the molecules remaining in culture fluid to nearly undetectable levels (Fig. 5B). These results are consistent with the idea that HMAQs and HMAQ-NOs are removed by separation of the cells and filtration of the remaining fluid, providing an explanation as to how the activity of these molecules has been missed in prior experiments.

HMAQ biosynthesis in *B. ambifaria*. The *Burkholderia ambifaria* genome contains an *hmq* operon homologous to that of *Burkholderia thailandensis* (21). However, *B. ambifaria* does not produce HMAQ-NOs (21), presumably because it does not have a

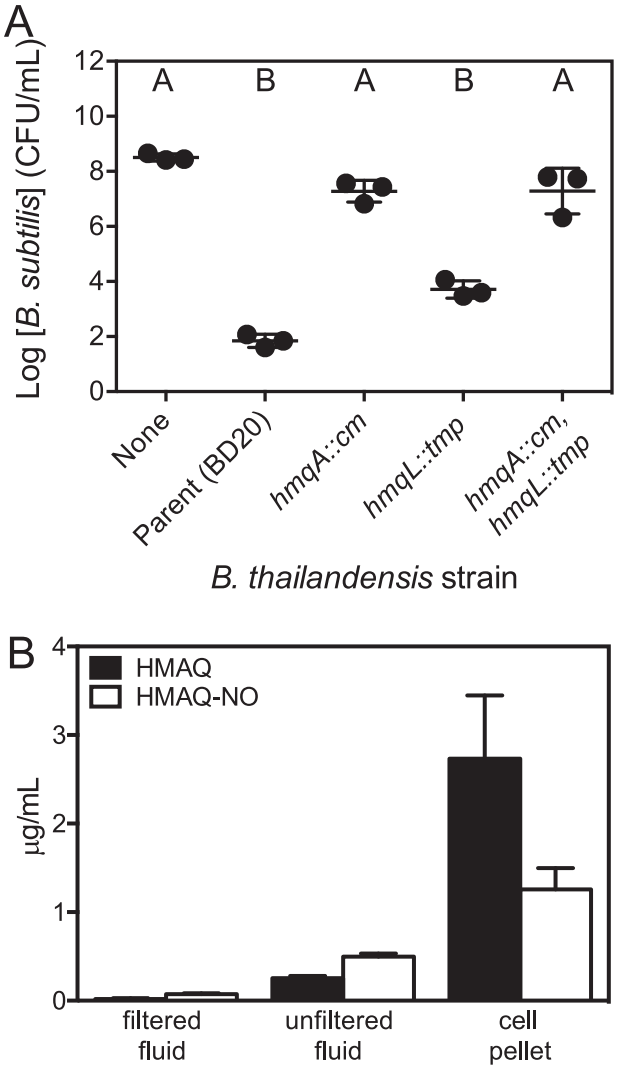

FIG 5 Involvement of BTH_II1576 (*hmqL*) in liquid cocultures and cell pellet fraction localization of HMAQ and HMAQ-NO molecules. (A) Results of cocultures of *B. subtilis* strain 168 combined in a 1:1 ratio with the bactobolin-deficient *B. thailandensis* (BD20) parent strain or the parent strain bearing a constructed disruption of *hmqA*, *hmqL*, or both in LB broth and grown for 24 h at 37°C. Surviving CFU were enumerated by serial dilution and plating on LB agar containing, for *B. subtilis*, 5% NaCl (nonpermissive for *B. thailandensis* growth), and for *B. thailandensis*, 100 μg/ml gentamicin (nonpermissive for *B. subtilis*). Data are representative of three biological replicates, and the error bars represent standard deviations. Statistical significance was determined by repeated measures one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison tests, and the letters A and B represent statistically significantly different groups ($P < 0.05$). (B) C_9 congeners of HMAQ and HMAQ-NO were quantified in unfiltered and filtered fluid from cell-free *B. thailandensis* cultures, as well as in pelleted cells, using LC-MS/MS and methods described previously (21). Error bars show standard deviations.

homolog of *hmqL* and *pqsL*. We predicted that introducing the *B. thailandensis hmqL* to *B. ambifaria* would enable production of HMAQ-NO. To test this prediction, we introduced the *hmqL* gene to *B. ambifaria* on plasmid pME6010 (33). Because HMAQ biosynthesis is less well characterized in this species, we used combined measurements of all three C_7, C_8, and C_9 congeners of HMAQs for our analysis. We observed that *B. ambifaria* with pME6010 had no detectable HMAQ-NO, as previously reported (21). However, *B. ambifaria* with pME6010-*hmqL* produced measurable levels of HMAQ-NO (Fig. 6A), which is consistent with the idea that HmqL is the only missing enzyme and its presence permits the production of HMAQ-NO production in *B. ambifaria*. This strain also had 100-fold less HMAQ than the empty plasmid-only strain (Fig. 6A), suggesting strong competition for the HMAQ precursor, likely 2-aminobenzoyl(methyl)acetate (30)

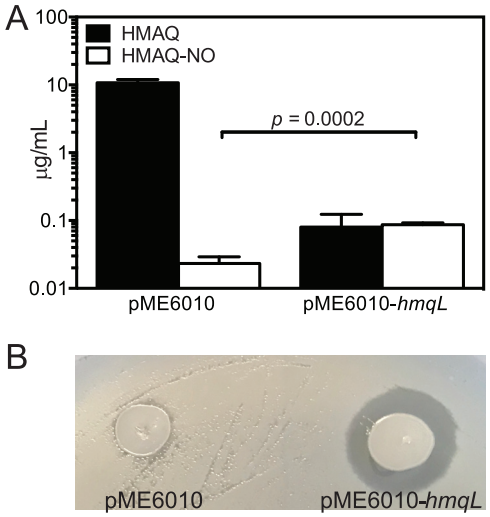

FIG 6 Heterologous expression of *hmqL* in *Burkholderia ambifaria*. (A) HMAQ and HMAQ-NO in cultures of *B. ambifaria* HSJ1 cells containing either pME6010 or pMP6010-*hmqL*. Results are the average of three biological replicates and represent the sum of the C_7, C_8, and C_9 congeners of each molecule. Error bars show standard deviations. (B) Antimicrobial activities of unfiltered fluid (5 μl) from cultures of *Burkholderia ambifaria* HSJ1 containing pME6010 or pME6010-*hmqL* spotted onto freshly spread lawns of *B. subtilis* on plates. Plates were imaged after 24 h of incubation at 37°C.

(the product of HmqADEG) (Fig. 3). We also tested whether the expression of HmqL caused *B. ambifaria* to inhibit *B. subtilis* growth. We spotted unfiltered culture fluid from *B. ambifaria* with pME6010 or pME6010-*hmqL* onto a lawn of *B. subtilis*. Only cultures of the strain expressing *hmqL* could inhibit *B. subtilis* growth (Fig. 6B). Together, the results provide further support for the idea that HmqL is crucial for production of the HMAQ-NO antimicrobials.

DISCUSSION

Investigation of the antimicrobial properties of HAQs dates back to 1945, when an "antibiotic metabolite" was described in *P. aeruginosa* (34). Although the biosynthesis steps and biology of the HAQs in *P. aeruginosa* have since been studied in detail, much less is known of those in *B. thailandensis* (21, 31, 35). The results of this study add new information to the known steps of biosynthesis of *B. thailandensis* HMAQs. Previous studies showed that enzymes analogous to PqsABCD in *P. aeruginosa* are involved in the synthesis of *B. thailandensis* HMAQ from anthranilate (Fig. 1, right column). In *P. aeruginosa*, the enzyme PqsL catalyzes an essential step in the synthesis of HAQ *N*-oxides (29, 30). *B. thailandensis* has no PqsH enzyme homolog and does not make 3-hydroxylated HAQs; a methyl is instead present as a substitution at that position. *B. thailandensis* is also missing a homolog of the HHQ/PQS receptor gene, *mvfR*. Our study validates the involvement of HmqL in *N*-oxide HMAQ biosynthesis and shows how the HMAQ family of molecules contribute to the arsenal of compounds used by *B. thailandensis* to compete with other species. The findings also provide new insight into the activities of specific *B. thailandensis* HMAQ family congeners against other bacteria.

Like many toxins, H(M)AQs have several known functions. In *P. aeruginosa*, where these molecules are best studied, the *N*-oxide congeners are potent antimicrobials that inhibit Gram-positive bacteria (24, 36, 37), and several of the HAQs are important for interspecies competition (38–40). Other *B. thailandensis* antimicrobials include bactobolin (12), malleilactone (14, 15), and thailandenes (18). This suite of diverse antimicrobials might be important for surviving competition with other microbes when space or other resources become limited. The loss of the *hmq* biosynthesis genes from the genome of the closely related host-adapted pathogen *B. mallei* supports a role of these genes in the saprophytic lifestyle of *B. thailandensis*. The current study demonstrates that the *N*-oxide HMAQs are important for antimicrobial activity toward other species

under several laboratory coculture conditions, similar to *P. aeruginosa* HQNO. HQNO also has other known effects, such as enhancing biofilm formation (41, 42) or increasing resistance to antimicrobials (43, 44), and it remains to be seen if HMAQ-NO is similar in these other ways.

We find it interesting that the *Burkholderia* species do not have the enzyme responsible for generating PQS (PqsH) (Fig. 1). PQS has a variety of known functions, such as immune modulation (45), cell density-dependent gene regulation (24, 46), and iron sequestration (26). *B. thailandensis* might have lost the ability to synthesize PQS because these functions are not needed or because there is existing functional redundancy with other molecules or pathways. For example, the small molecule malleilactone might have similar biophysical properties and also sequester iron (14). It is also interesting that *B. ambifaria* lacks the HmqL enzyme responsible for generating *N*-oxide HMAQs, which are the most antimicrobial members of this family. The lack of PQS or any *N*-oxide analog in *B. ambifaria* strongly supports the idea that other products of this pathway have important functions that contribute to the survival of this species, although the biology of the other products of the Hmq system is not well understood.

A particularly interesting discovery in this work was that *B. thailandensis* HMAQ-NO-$C_{9:2'}$ is much more active (33-fold) than *P. aeruginosa* HQNO (HAQ-NO-C_7) against *B. subtilis* (Table 2). The heightened activity of HMAQ-NO compared with that of HQNO could be due to both side chain length and, possibly, methylation (or saturation). It remains to be seen whether the structural moieties important for this activity alter the target site of this molecule, the ability to penetrate *B. subtilis* cells, or some other aspect of this molecule. In addition to the *N*-oxide congeners, *B. thailandensis* produces a variety of HMAQs with side chains of various lengths and degrees of saturation (21, 26). Although these other molecules had less potent antimicrobial activities (Table 2), it is possible they contribute to competition in other ways. A previous study showed that different variations of HAQs used in combination can have synergistic antimicrobial effects on other bacteria by acting on distinctly different cellular targets (47). Thus, the diversity of H(M)AQs produced by *B. thailandensis* might serve to enhance activity against competitors or could be important for averting the development of antibiotic resistance in competitors.

Our findings that HMAQs and HMAQ-NOs are associated with the cell and are particularly potent toward *B. subtilis* explain how their antimicrobial activity was missed in prior studies (11, 48–50). *P. aeruginosa* HAQs are similarly cell associated (26). Given the antibacterial properties of HMAQ-NOs and HQNO, it is curious that these molecules are cell associated. It may be that their antimicrobial activity is important under conditions, such as biofilms, where close contact with other cells occurs, rendering it beneficial for the activity to remain associated with the cell surface. An alternative possibility is that their primary role involves another function that is needed at the cell surface. For example, the role of PQS in iron entrapment requires an interaction with the cell surface (26). It is also noteworthy that HMAQ-NOs, along with other antimicrobials produced by *B. thailandensis*, can be associated with outer membrane vesicles (OMVs) (51). OMVs are buds of the outer membrane that are released from the cell (52). *B. thailandensis* OMVs have antimicrobial properties (51), similar to OMVs of other Gram-negative bacteria (53). OMVs fuse with the cell membrane of a competitor to deliver toxins directly into the periplasm (53). Thus, antimicrobials associated with the cell membrane are likely to be packaged into OMVs, which can enhance their activity during competition.

MATERIALS AND METHODS

Bacterial culture conditions and reagents. Bacteria were grown in lysogeny broth (LB) (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter), supplemented with 50 mM MOPS (morpholinepropanesulfonic acid) pH 7 in M9 minimal medium supplemented with 0.4% D-glucose and 10 mM *para*-chloro-phenylalanine (*p*-Cl-Phe; Sigma) for *B. thailandensis* counterselection during mutant construction or in DM medium (0.25× M63 salts, 1 mM $MgSO_4$, 0.4% glycerol, 0.2% glucose, 1 μg/ml thiamine, and 40 μg/ml each of leucine, isoleucine, valine, tryptophan, glutamic acid, and glutamine) for transformation of PCR-generated products. For liquid cocultures, *B. subtilis* and *B. thailandensis* growth was at 37°C. For all other experiments, *B. thailandensis* growth was at 30°C, and all *E. coli* and *B. ambifaria*

TABLE 3 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
Burkholderia thailandensis strains		
E264	Wild type	5
BD20	E264 with deletion of *btaK*	11
JRK100	E264 *hmqA*::*cm*	This study
JRK101	E264 BTH_II1576::FRT-*tmp*	This study
JRK102	BD20 *hmqA*::*cm*	This study
JRK103	BD20 BTH_II1576::FRT-*tmp*	This study
JRK104	BD20 *glmS1 attn7*::P*lac*; Km^r	This study
JRK105	JRK103 *glmS1 attn7*::P*lac*; Km^r	This study
JRK106	JRK103 *glmS1 attn7*::P*lac-hmqL*; Km^r	This study
Escherichia coli strains		
DH5α	F$^-$ ϕ80*lacZ* ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17*(r_K^- m_K^+) *recA1 endA1 phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
JM109	(*traD36 proAB*$^+$ *lacI*^{q} *lacZ* M15) *endA1 recA1 hsdR17*(r_K^- m_K^+) *mcrA supE44* λ^- *gyrA96 relA1* (*lac proAB*)	67
Other strains		
Bacillus subtilis 168	Wild type	68
Staphylococcus aureus Newman	Wild type	57
Pseudomonas aeruginosa PA14	Wild type	58
Burkholderia ambifaria HSJ1	Wild type	21
Plasmids		
pJRC125	Suicide plasmid; Tp^r	6
pMCG19	pEX18Tp-PheS with an *hmqA* disrupted by a chloramphenicol resistance cassette (*hmqA*::*cm*)	This study
pTNS2	Tn*7* transposase-expressing helper plasmid; Amp^r	69
pUC18miniTn7T-P*lac-malR*	Mobilizable mini-Tn*7* vector with the *lac* promoter (P*lac*) for IPTG-inducible *malR* expression (used to construct pUC18-mini-Tn*7*T-P*lac-malR*); Km^r, Ap^r	61
pUC18miniTn7T-P*lac-hmqL*	pUC18miniTn7T-LAC-Km containing the BTH_II1576 gene (*hmqL*); Km^r, Ap^r	This study
pME6010	pVS1-p15A shuttle vector; Tc^r	33
pME6010-*hmqL*	pME6010 with the *B. thailandensis* BTH_II1576 (*hmqL*) gene	This study

growth was at 37°C. 4-Hydroxy-2-heptylquinoline (HHQ) was purchased from Sigma (catalog number SML0747). 4-Hydroxy-2-heptylquinoline *N*-oxide (HQNO) was purchased from Cayman Chemicals (catalog number 15159). 4-Hydroxy-3-methyl-2-nonenylquinoline (HMNQ) was purified from *B. thailandensis* E264 cultures as described previously (21). The other hydroxyalkenylquinolines were synthesized as described below. For selection, trimethoprim was used at 100 μg/ml, gentamicin was used at 100 μg/ml, kanamycin was used at 500 μg/ml (*B. thailandensis*) or 50 μg/ml (*E. coli*), tetracycline was used at 225 μg/ml (*B. ambifaria*), and NaCl was used at 5% (for inhibiting *B. thailandensis* in coculture enumerations). IPTG (isopropyl β-D-1-thiogalactopyranoside) was added at 1 mM final concentration to cultures and plates, when appropriate. Genomic DNA, PCR and DNA fragments, and plasmid DNA were purified using a Puregene core A kit, plasmid purification miniprep kit, or PCR cleanup/gel extraction kits (Qiagen or IBI-MidSci) according to the manufacturer's protocol.

Synthesis of *N*-oxides of hydroxyalkenylquinolines. HMAQ-NOs were synthesized as previously described (31) from corresponding HMAQs in which the quinolone scaffold was built via the Conrad-Limpach approach (54). Briefly, aniline was condensed with diethyl 2-methyl-3-oxosuccinate and the resulting diester was cyclized under acidic conditions. Reduction of the quinolone ester followed by halogen substitution led to 2-chloromethyl-3-methylquinolin-4(1*H*)-ones, which were subjected to Suzuki-Miyaura cross-coupling (55) with commercially available alkenylboronic acid pinacol esters to provide HMAQs. Then, they were converted into the corresponding ethyl carbonates, oxidized with *meta*-chloroperbenzoic acid (mCPBA), and deprotected to yield HMAQ-NOs (56). The structures of HMAQ-NOs were confirmed by high-resolution mass spectrometry, as well as 1-dimensional (1-D) and 2-dimensional (2-D) nuclear magnetic resonance (NMR) analysis. Structural data are available in Piochon et al. (31).

Bacterial strains and genetic manipulations. All bacterial strains, plasmids, and primers used in this study are listed in Tables 3 and 4. We used the wild type and mutant derivatives of *B. thailandensis* strain E264 (5). We used *B. subtilis* strain 168 (53), *S. aureus* strain Newman (57), *P. aeruginosa* strain PA14 (58), *B. ambifaria* strain HSJ1 (21), and *E. coli* strain DH5α for genetic manipulations (Invitrogen). The *B. thailandensis* bactobolin-defective mutant BD20 has a deletion of the bactobolin biosynthesis gene *btaK* as described previously (11). The *B. thailandensis hmqA* mutant was constructed by allelic exchange using methods described previously (6) and plasmid pMCG19. pMCG19 was constructed by first amplifying *hmqA* from the *B. thailandensis* E264 genome using primers hmqAfor and hmqArev containing HindIII and KpnI cleavage sites, respectively. The PCR product was digested with HindIII and KpnI and ligated to

TABLE 4 Primers used in this study

Purpose, primer	Sequence (5′ to 3′)
Tn*5* mutant identification	
hmqA F-1	GATCTGCCATTGCTTTCCGCAACACG
hmqA R-1	TCAGGCCGCTTGCACGTCG
hmqF F-1	GCTGCATCTGAAGAGCATGGAGC
hmqF R-1	CGTGCTCTCTTCGTGATATCCCATCC
hmqC F-1	TCGGCAATGTGCGAAGCAAGGTC
hmqC R-1	GAGCGGATTGTCGGCAACGAC
hmqL-Tn*5*-Tp-F2	CGTCATGCCCAATGTGCGCTTG
hmqL-Tn*5*-Tp-R2	GTTGGTTGACGACTGCGCGAAC
Constructing pUC18miniTn7T-P*lac-hmqL* (BTH_II1576)	
hmqL-ORF-F-SacI	ATATTAGAGCTCATGAAAAACAACCAAGTCGATG
hmqL-ORF-R-HindIII	ATATTAAAGCTTATTCCCGCTTCGTCCGCCAGC
Constructing pMCG19 (*hmqA*::*cm*)	
hmqAfor	ACGAAGCTTCATCTCTTGCCGCAGCTTGAA
hmqArev	ACGGTACCGATCATCAGCCTCGGCTACAC
CmFPstI	AAAACTGCAGGTGACGGAAGATCACTTCGCA
CmRPstI	AAAACTGCAGGCGTTTAAGGTCAACAATAACTGC
Constructing pME6010 *hmqL*	
hmqL-F	CCGAGATCTACCCAATTCATAGACCAGCGTTGC
hmqL-R	CCGGGTACCTCATGATGCGTACCTCCGTCGATT
Constructing the *hmqL*::*dhfR* mutant by natural transformation	
hmqL-Tn-for2	CGTCATGCCCAATGTGCGCTTG
hmqL-Tn-rev2	GTTGGTTGACGACTGCGCGAAC

HindIII-KpnI-cut pEX18Tp-PheS (9). The chloramphenicol resistance cassette was amplified from pACYC184 (59) using primers CmFPstI and CmRPstI, each containing the PstI cleavage site, and ligated to the PstI site inside the *hmqA* gene in pEX18Tp-PheS-*hmqA* to make pMCG19.

B. thailandensis BTH_II1576 (*hmqL*) mutants were made by transforming a PCR-amplified BTH_II1576::*dhfR* allele from transposon mutant 56 into the genome of strain BD20 using PCR transformation with a modified protocol similar to that of Thongdee et al. (60). Briefly, shaking *B. thailandensis* cultures were grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.5, concentrated 20-fold, and distributed to five aliquots of 50 μl. Each aliquot was mixed with 5 μl of gel-extracted *hmqL*::*dhfR* PCR product (amplified using hmqL-Tn-for2 and hmqL-Tn-rev2 primers). The cell-DNA mixture was spotted onto solid DM medium (DM liquid medium with 1.5% agar) and incubated at 37°C for 48 h. The DM plate growth was scraped up and collected, washed twice with DM, suspended in 200 μl DM, and spread onto LB agar containing trimethoprim. Mutant strains were verified by PCR amplifying the mutated region and sequencing the PCR product.

For ectopic expression of *hmqL* in *B. thailandensis*, this gene was placed under the control of the IPTG-inducible *lac* promoter in pUC18miniTn7T-LAC-Km (61). To construct this plasmid, we amplified *hmqL* from the *B. thailandensis* E264 genome using primers hmqL-ORF-F-SacI and hmqL-ORF-R-HindIII that incorporated the SacI and HindIII restriction enzyme sites, respectively, into the product. The amplicon was cut with SacI and HindIII and ligated to SacI- and HindIII-digested pUC18miniTn7T-Kan-P*lac-malR* (61) to make pUC18miniTn7T-P*lac-hmqL* (entirely removing the *malR* gene). This plasmid was used to transform competent *B. thailandensis* cells with the helper plasmid pTNS2 as described previously (62). We used PCR to verify insertion of the P*lac-hmqL* cassette into the *attn7* site near *glmS1*.

We used plasmid pME6010 (33) for expressing the *hmqL* gene from *B. thailandensis* in *B. ambifaria*. The *hmqL* (BTH_II1576) gene was amplified from the *B. thailandensis* E264 genome using primers hmqL-F and hmqL-R that incorporated the BglII and KpnI sites into the amplicon. The product was cut with BglII and KpnI and ligated to BglII- and KpnI-digested pME6010 to make pMCG17. *B. ambifaria* strains with pME6010 plasmids were constructed by electroporation as previously described for *B. thailandensis* (6).

Liquid cocultures. Logarithmic-phase overnight starter cultures (OD_{600} between 0.5 and 1.5) of *B. subtilis* and *B. thailandensis* were diluted to an OD_{600} of 0.05 and combined at a starting ratio of 1:1 in a 10-ml volume of LB in 125-ml baffled flasks. The flasks were incubated with shaking at 250 rpm at 37°C for 24 h before serially diluting and plating on LB agar plates containing gentamicin (to inhibit *B. subtilis*) or 5% NaCl (to inhibit *B. thailandensis*) and IPTG as appropriate to enumerate bacterial CFU.

Antimicrobial activity assays. Antimicrobial activities of *B. thailandensis* culture fluid were assayed using disc diffusion (for filtered fluid) or outgrowth diffusion (for unclarified fluid) methods. For both methods, inocula for each of the *B. thailandensis* strains and *B. subtilis* were prepared by suspending a colony from an LB agar plate into LB broth and growing overnight at 30°C with shaking. *B. subtilis* overnight culture (100 μl) diluted 1:100 was spread onto LB agar plates and allowed to dry. Filter discs

were placed on the *B. subtilis* lawns and saturated with *B. thailandensis* cultures that were either centrifuged and filter sterilized through a 0.2-μm membrane (for disc diffusion) or spotted directly onto the *B. subtilis* lawns (for outgrowth diffusion). The plates were incubated at 30°C for 24 h before observing zones of clearing of the *B. subtilis* lawns. The outgrowth assays were also conducted similarly on LB agar plates containing 5% NaCl, which inhibits growth of the *B. thailandensis* strains.

The antimicrobial activities of purified, commercial, or synthesized hydroxyalkylquinoline compounds were assessed using a MIC assay according to a protocol modified from the 2020 guidelines of the Clinical and Laboratory Standards Institute (CLSI) (70). Inocula for each test organism were prepared by suspending a colony from an LB agar plate into tryptic soy broth (TSB) and growing for 3 to 5 h at 37°C with shaking, then adjusting the culture turbidity in TSB to an OD_{600} of 0.25, roughly the equivalent of a 1.0 McFarland standard (3×10^8 CFU per ml). These cell suspensions were used as inocula for microtiter MIC assays. A 2.5-μl inoculum, which corresponded to 1×10^6 cells, was added to a 100-μl well containing diluted cation-adjusted Mueller-Hinton II broth, and these were incubated with shaking for 24 h at 37°C. The MIC was defined as the lowest concentration of compound (μg/ml) with which bacterial growth in the well was not visible.

Transposon mutagenesis and screen. Transposon mutagenesis was performed using the EZ-Tn5 <DHFR-1>Tnp Transposome kit (Epicentre) according to the manufacturer's specifications. Briefly, electrocompetent cells of the *B. thailandensis* bactobolin-defective mutant BD20 were generated by growing cultures to mid-exponential phase (OD_{600} of 0.5 to 0.7), collecting them with centrifugation, washing the cell pellet three times in ice-cold 0.5 M sucrose (using 25% the volume of the original culture), and then resuspending the cell pellet in 100 μl ice-cold 0.5 M sucrose. Immediately, 1 μl transposome (the EZ-Tn*5* transposon plus the needed transposase for transposition) was added to 50 μl electrocompetent cells in a 0.2-mm electroporation cuvette. This was electroporated with the Bio-Rad Gene Pulser II (using settings of 25 μF, 200 Ω, and 2.5 kV), and the cells were immediately recovered in 1 ml LB broth with shaking at 37°C for 1 h. At the end of the recovery, the culture was diluted 1:25, and 100-μl samples were plated on 20 LB plates with trimethoprim selection (100 μg/ml). The plates were incubated overnight at 37°C. The following day, single colonies were patched onto plates prepared with *B. subtilis* to screen for antimicrobial activity. Due to the scale required for the screen, we added *B. subtilis* directly to molten agar used to pour plates, as opposed to spreading *B. subtilis* lawns after pouring. To prepare the *B. subtilis*-agar medium, we added 1.43 ml of a stationary-phase *B. subtilis* culture (overnight growth) to 1 liter of cooled but molten LB agar medium (55 to 60°C), mixed gently, and poured. After a brief period to solidify and dry, plates were used to patch colonies isolated from the EZ-Tn5 <DHFR-1> transposon mutagenesis. Patched plates were incubated overnight at 30°C prior to identifying mutants defective for antimicrobial activity, as determined by reduced zones of *B. subtilis* growth inhibition compared with the growth of the *B. thailandensis* parent. Identified candidates were streaked for single *B. thailandensis* colonies on LB with gentamicin to prevent *B. subtilis* growth and retested in our assay to confirm the phenotype. Confirmed mutants with no apparent growth defects were subjected to whole-genome sequencing.

Identification of transposon insertion sites. The transposon insertion locations of five transposon mutants (mutants 7, 14, 31, 32, and 56) were determined by whole-genome resequencing. DNA isolated from the transposon mutant strains was used to make sequencing libraries with 300-bp inserts. The libraries were sequenced on an Illumina MiSeq system using the NEBNext ultra II kit, generating approximately 1 million 200-bp paired-end reads per sample. The paired-end reads were assembled *de novo* into draft genomes using the SPAdes assembler with standard settings (63). For each *de novo* assembly, the contig with the Tn*5* transposon sequence was located using a nucleotide search in the BLAST+ command line suite with individual blast databases for each transposon mutant (64). Clustal Omega was then used to precisely locate the sequence context of Tn*5* insertion in each contig of interest (65). The genomic context for individual transposon insertions was then determined by blasting up- and downstream sequences against a database of all *B. thailandensis* E264 gene sequences to identify specific loci interrupted by Tn*5* insertion. Finally, the raw reads were aligned to the *B. thailandensis* E264 ATCC 700388 reference genome (accession numbers NC_007650 and NC_007651, downloaded from https://www.burkholderia.com) using Strand NGS (Bangalore, India) software version 3.1.1 to confirm the insertion locus in each mutant. The remaining four transposon mutants (mutants 9, 27, 63, and 68) were assessed by PCR amplifying regions of the *hmq* locus (primers are given in Table S2 in the supplemental material). Mutations identified by either method were verified by Sanger sequencing of PCR-amplified products.

HMAQ and HMAQ-NO measurements from bacterial cultures. To measure the production of HMAQ and HMAQ-NO in *B. thailandensis* cultures, samples were prepared by diluting stationary-phase *B. thailandensis* cultures to an OD_{600} of 0.05 into 5 ml of LB in 18-mm culture tubes and growing the cultures for 18 h with shaking at 250 rpm at 30°C. Where necessary, 1 mM IPTG was added to the LB at the beginning of the growth experiment. At 18 h, sample preparation and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed as described by Lépine et al. (66), with minor modifications. Briefly, for each sample, 300 μl of grown culture was mixed with 300 μl of high-performance liquid chromatography (HPLC)-grade methanol containing 4 ppm of 5,6,7,8-tetradeutero-4-hydroxy-2-heptylquinoline (HHQ-d_4) as an internal standard, vortexed, and centrifuged for 5 min at maximum speed in a microcentrifuge. The supernatant-methanol solution was carefully recovered for analysis. Samples were analyzed by HPLC (Waters 2795; Waters, Mississauga, ON, Canada) equipped with a Kinetex (100- by 3.0-mm) 5-μm EVO C_{18} reverse-phase LC column (Phenomenex). The detector was a tandem quadrupole mass spectrometer (Quattro premier XE; Waters) equipped with a Z-spray interface using electrospray ionization in positive mode (ESI+). The capillary voltage was set at

3.0 kV, and the cone voltage at 21 V. The source temperature was kept at 120°C. Nitrogen was used as a nebulizing and drying gas at flow rates of 15 and 100 ml · min^{-1}, respectively. Data were collected in scan mode from 130 to 530 Da. An acetonitrile-water gradient containing 1% acetic acid was used. The HPLC flow rate was 400 μl · min^{-1} split to 40 μl · min^{-1} by a Valco tee splitter. Quantification of all the HMAQs and HMAQ-NOs was performed using the response factor of 4-hydroxy-3-methyl-2-heptenylquinoline (HMAQ-C_7) and using HHQ-d_4 as an internal standard.

Data availability. All strains, plasmids, and sequence data generated by these studies will be made available upon request (Chandler Laboratory [jrchandler@ku.edu], University of Kansas).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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